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Xylella fastidiosa subspecies: *X. fastidiosa* subsp *piercei*, subsp. nov., *X. fastidiosa* subsp. *multiplex* subsp. nov., and *X. fastidiosa* subsp. *pauca* subsp. nov.

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Summary

Xylella fastidiosa, a fastidious bacterium causing disease in over 100 plant species, is classified as a single species, although genetic studies support multiple taxons. To determine the taxonomic relatedness among strains of *X. fastidiosa*, we conducted DNA-DNA relatedness assays and sequenced the 16S–23S intergenic spacer (ITS) region using 26 strains from 10 hosts. Under stringent conditions (T_m –15 °C), the DNA relatedness for most *X. fastidiosa* strains was ≥70%. However, at high stringency (T_m –8 °C), three distinct genotypes (A, B, and C) were revealed. Taxon A included strains from cultivated grape, alfalfa, almond (two), and maple, interrelated by 85% (mean); taxon B included strains from peach, elm, plum, pigeon grape, sycamore, and almond (one), interrelated by 84%; and taxon C included only strains from citrus, interrelated by 87%. The mean reciprocal relatedness between taxons A and B, A and C, and B and C, were 58, 41, and 45%, respectively. ITS results also indicated the same grouping ; taxons A and B, A and C, and B and C had identities of 98.7, 97.9, and 99.2%, respectively. Previous and present phenotypic data supports the molecular data. Taxon A strains grow faster on Pierce's disease agar medium whereas B and C strains grow more slowly. Taxon B and C strains are susceptible to penicillin and resistant to carbenicillin whereas A strains are opposite. Each taxon can be differentiated serologically as well as by structural proteins. We propose taxons A, B, and C be named *X. fastidiosa* subsp. *piercei*, subsp. nov, subsp. *multiplex*, subsp. nov., and subsp. *pauca*, subsp. nov., respectively. The type strains of the subspecies are subsp. *piercei* ICPB 50025 (=ATTC 35879^T and ICMP 15197), subsp. *multiplex* ICPB 50039 (= ATTC 35871 and ICMP 15199), and subsp. *pauca* ICPB 50031 (= ICMP 15198).

Key words: DNA-DNA hybridization – 16S–23S ITS sequencing – fastidious plant pathogenic bacterium

Introduction

Xylella fastidiosa, Wells et al. [80], is emerging as a very destructive pathogen on numerous plant hosts in North and South America [12, 33, 42, 53, 60, 63, 69]. Most plant pathogenic bacteria are somewhat host specific, often causing disease in plants of a single family [7], however, *X. fastidiosa* has a very wide host range causing disease in over 100 species of plants from at least 46 plant families [http://www.cnr.berkeley.edu/xylella/temp/hosts.htm, 42, 66]. The first report of a disease caused by *X. fastidiosa* was California vine disease of grape (*Vitis vinifera* L.) in Southern California in 1892 [64]. The disease, eventually known as Pierce's disease, spread to other grape growing areas in California, and was shown to be transmitted by leafhopper insects [30, 35, 36]. The causal agent has been described variously as a virus [29, 43], a rickettsia-like bacterium [32, 41, 51], and a Gram-positive bacterium, *Lactobacillus hordiniae* [3]. Not until the fastidious organism was shown to be a thin, rod-shaped, Gram-negative bacterium and Koch's postulates completed was the actual pathogen described [22]. In 1987, Wells et al. [80] proposed a new genus, *Xylella*, and named the organism *X. fastidiosa* based on its fastidious growth. Although *X. fastidiosa* infects many plant species, it is often considered as a weak or opportunistic pathogen. Marginal scorching of leaves is the most common

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symptom caused by *X. fastidiosa*, but it is not normally present until late in the season when temperatures are high and plants are water-stressed [36, 38, 39]. Wine grapes often show severe symptoms and die late in the season because the vines are purposely water-stressed prior to harvest as part of routine viticultural practices for making quality wine. Diseased plants, especially shade trees, are often characterized by delayed bud break, reduced growth, leaf scorch, and dieback, especially during droughty fall months [73]. Pierce's disease is currently causing severe losses in grapes in California and Texas, and citrus variegated chlorosis, also caused by *X. fastidiosa*, is causing severe losses in oranges (*Citrus* spp.) in Brazil.

The scorching symptoms so commonly induced by *X. fastidiosa* are a result of the plugging of the vascular system [28] and are easily confused with other factors such as drought, salt toxicity, or herbicide injury [62]. Geographical distribution of diseases caused by *X. fastidiosa* is limited to warmer areas of North and South America except for a report of pear (*Pyrus communis* L.) leaf scorch in Taiwan [55]. Unconfirmed reports of diseases caused by *X. fastidiosa* in other locations include Pierce's disease in Yugoslavia [6] and almond (*Prunus persica* (L.) Batsch) leaf scorch in India [44]. Several molecular studies have shown enough genetic variability within *X. fastidiosa* to justify separate taxons [20, 34, 49, 65] but no formal phylogenetic proposals have been made for further speciation or sub-speciation. In this report, we examine possible phylogenetic groups among 26 *X. fastidiosa* strains isolated from 10 plant species using DNA-DNA relatedness and 16S–23S intergenic spacer (ITS) sequence assays. DNA relatedness assays are the standard for determining phylogenetic relationships of bacteria at the species level [75, 76, 77]. Sequence analysis of the ITS region contains considerable variation and has proven useful for determining phylogentic relatedness at the species level [5] and considerable sequence data is available for comparison. On the basis of these and published data, we propose that the 26 strains be classified into three subspecies, *X. fastidiosa* subsp. *piercei*, *multiplex*, and *pauca*.

Materials and Methods

Bacterial strains

Strains of *X. fastidiosa* were obtained from several sources, including two original strains for this study (Table 1). A total of 26 strains from 10 hosts were used. Cultures were maintained for routine use by monthly transfers on periwinkle wilt (PW) [21] or Pierce's disease 2 (PD2) [23] agar. For long-term storage, each strain was grown in liquid media, either PD2 or PW, at 26–28 °C on a rotary shaker for 14–20 days, centrifuged, resuspended in 15% glycerol, and kept in vials at –80 °C. Just prior to DNA extraction, all strains were cloned on agar media, PD2 and/or PW, and their identity confirmed by phase contrast microscopy and real-time PCR using X. *fastidiosa*-specific primers and probe [70].

DNA extraction. For DNA-DNA relatedness assays, only DNAs with 260/280 ratios of 1.8 to 1.9 were used. DNA was extracted from cells by a modified method of Marmur [47, 58, 68, G. Lacy, unpublished]. Briefly, cells were grown in one liter of liquid PW or PD2 at 26–28 °C on a rotary shaker for 14–20 days. To confirm the culture had not become contaminated, streaks were made onto yeast extract-dextrose $CaCO_3$ (YDC) [82], PW, and PD2 agar plates to check for lack of growth on YDC and for presence of typical small (0.5–1 mm diameter) "fried egg-like" colonies on PW or PD2 agar after 7–10 days. The cells were washed by centrifugation and suspended in TES buffer (10 mM Tris-HCL, 1 mM EDTA, 0.35 M sucrose, pH 8.0) and frozen until purity was confirmed. The cells were thawed and peptidglycans degraded by adding 0.1 mg/ml of lysozyme and incubating for one hour at 37 °C. After adding one volume of 5 M sodium perchlorate, two volumes of lysing solution (100 mM Tris-HCl, 0.3 M NaCl, 20 mM EDTA, 2% (w/v) SDS, pH 8.0), 100 ug/ml of proteinase K, and 2% β-mercaptoethanol (v/v), the crude extract was incubated for one-two hours in a water bath at 55–60 °C to denature proteins. Thirty ml of phenol: sevag (chloroform: isoamyl alcohol, 24:1 v/v) at a 1:1 v/v ratio was added and the mixture shaken vigorously for 20–30 min. The resulting suspension was centrifuged at 17,000 × g for 10 min at 4 °C and the aqueous layer containing DNA carefully removed with a large bore pipette and transferred into a new centrifuge tube and the centrifugation step repeated. The DNA was precipitated with 0.6 vol 99% isopropanol, washed twice in 76% ethyl alcohol to remove any salts and dried. After dissolving in 20 ml of TE buffer (10 mM Tris-HCl 1 mM EDTA, pH 8.0), RNA was degraded by adding 0.25 ml of a RNase mixture [29.4ul of RNase A (Sigma R-4642) and 3.6ul of T_1RNase (Gibco/BRL 18030-015) in 970ul of TE buffer] and the mixture incubated at 37 °C for one hour. Proteins were denatured by adding 15 ml of sevag and centrifuging at 17,000 × g for 10 min at 4 °C. After washing twice in 76% ethanol, the DNA was precipitated by adding two volumes of 95% ethanol and dissolved in 4 ml of TE buffer. The purified DNA was stored at –20 °C after determining the absorbance at 260/280 using a spectrophotometer (Perkin Elmer, San Jose, CA).

DNA–DNA relatedness

DNA relatedness assays were performed using a modified S_1 nuclease technique [46, 68]. After adjusting the concentration to 200 ng/ul, the DNA was sheared by passing three times through a French Pressure cell (Spectronic Unicam, Rochester, NY) at 1057–1409 kg/cm^2 (15,000–20,000 psi). The sheared DNA was denatured by boiling for 5 minutes and chilled in an ice-water slurry (0 °C) for 5 minutes to prevent duplex re-formation and stored at –20 °C. For labeled DNA, alpha-^{33}P deoxycytidine triphosphate (Amersham Bioscience, catalog 9905) was used with a Rad Prime DNA Labeling System kit (Invitrogen Life Technologies, Rockville, MD; Catalog No. 18428-011) except 5,000 ng of DNA was used and incubation was for 15 min without a stop buffer. Following labeling, the DNA was purified with NAP-25 columns (Amersham Bioscience, catalog 17-0852-01) as recommended using TE +0.1% SDS to eluate the DNA. The synthesized double-stranded DNA (dsDNA) was denatured to single-stranded DNA (ssDNA) by boiling. To prevent self reassociation, labeled ssDNA (10–20 µl) was diluted into non-labeled ssDNA at an excess concentration ratio of 500:1. *X. fastidiosa* DNA melting temperature (T_m = 90.4 °C) was determined from the mole percent guanine plus cytosine (% G+C) of 52.1% [79] using the formula of Marmur and Doty [59] [$T_m = (X_{\%G+C})\ (1.10) + (X_{\%A+T})\ (0.69)$]. For DNA reassociation temperatures (T_r) for species-level phylogenetic relationships, we used T_m –15 °C (T_{spp} = 75.4 °C), the most stringent temperature recommended for reassociation [45]. For subspecies level assays, we reassociated DNAs at even higher stringency ($T_{sub} = T_m$ –8 °C = 82.4 °C). T_{sub} was determined empirically based on observations that hybrid dsDNA duplexes formed between heterologous ssDNAs are less stable than duplexes formed by reassocia-

Table 1. Source of strains of *Xylella fastidiosa*.

Strain designation[a]	Host plant and geographic origins of isolation		Original strain designation and source	
	Host	Location	Original strain designations[b]	Source
50023	Grape (*Vitis vinifera* L.)	Georgia, USA	R3V18	C.J.Chang, University of Georgia, USA
50025	Grape	Florida, USA	ATCC 35879^T, 2683 PCE-RR	C.J.Chang, University of Georgia, USA
50028	Grape	Florida, USA	PD92-6	D.Hopkins, University of Florida, USA
50030	Grape	Florida, USA	PD 94-4	C.J.Chang, University of Georgia, USA
50035	Grape	Georgia, USA	PD95-3	C.J.Chang, University of Georgia, USA
50036	Grape	Florida, USA	ATCC 35881, 2694 PCE-FG	C.J.Chang, University of Georgia, USA
50040	Grape	California, USA	Stags Leap	S. Purcell, University of California, Berkeley, USA
50043	Grape	California, USA	Traver	S. Purcell, University of California, Berkeley, USA
50047	Grape	California, USA	93-1F	Original isolation made during this study
50033	Almond (*Prunus dulcis* (Mill.) Webb	California, USA	ATCC 35870, 2685 ALS-BC	D. Hopkins, University of Florida, USA
50045	Almond	California, USA	AC-8	D. Hopkins, University of Florida, USA (from M. Davis, University Florida)
50046	Almond	California, USA	ALS#1	S. Purcell, University of California, Berkeley
50037	Alfalfa (*Medicago sativa* L.)	California, USA	MT1	D. Hopkins, University of Florida, USA (from M. Davis, University of Florida)
50038	Alfalfa	California, USA	MT5G	D. Hopkins, University of Florida, USA (from M. Davis, University of Florida)
50068	Alfalfa	California, USA	MT3T	D. Hopkins, University of Florida, USA (from M. Davis, University of Florida)
50056	Maple (*Acer* spp.)	California, USA	Alameda	S. Purcell, University of California, Berkeley
50016	Plum, (*Prunus domestica* L.)	Georgia, USA	Plum 2#6	D. Hopkins, University of Florida, USA
50039	Hybrid plum	Georgia, USA	ATCC 35871, PL 788, 2679 PLM G83	D. Hopkins, University of Florida, USA
50032	Peach (*Prunus persica* L. Batsch)	Florida, USA	Peach 4#5	C.J.Chang, University of Georgia, USA
50063	American Elm (*Ulmus americana* L.)	Florida, USA	ATCC 35873	D.Hopkins, University of Florida, USA
50054	Pigeon grape (*V. aestivalis* Michx.)	Washington, D.C.	WGF#1	Original isolation made during this study
50059	Sycamore (*Platanus* spp.)	Washington, D.C.	#85	J.Sherald, National Park Service, Washington, D.C.
50024	Citrus (*Citrus* spp.)	Brazil	CVC 03-06	L.E.A. Camargo, Univ. Sao Paulo, Sao Paulo, Brazil
50031	Citrus	Brazil	CVC 09-02N	L.E.A. Camargo, Univ. Sao Paulo, Sao Paulo, Brazil
50066	Citrus	Brazil	CVC 08-01F	L.E.A. Camargo, Univ. Sao Paulo, Sao Paulo, Brazil
50082	Citrus	Brazil	IBSBF 1378	L.E.A. Camargo, Univ. Sao Paulo, Sao Paulo, Brazil

[a]International Collection of Phytopathogenic Bacteria (ICPB) maintained by ARS-USDA, Foreign Disease-Weed Science Research Unit, Frederick, MD.
[b]Abbreviations: ATCC, American Type Culture Collection, Manassas, VA.

tion of homologous ssDNAs [8, 9,10, 11, 48]. DNA was reassociated in reassociation buffer (5.28 M NaCl, 10 mM HEPES, 2.5 mM EDTA, pH 7.0) containing 22.7% formamide [47]. Because each 1% of formamide allows for a decrease in the reassociation temperature of 0.6 °C, the actual temperature of reassociation used was (22.7% X 0.6 °C = 13.6 °C; T_{spp} = 75.4 –13.6 °C = 61.8 °C and T_{sub} = 82.4 – 13.6 = 68.8 °C). All DNA-DNA reassociations were carried out in a water bath at either T_{spp} ± 0.5 °C or T_{sub} ± 0.5 °C for 24hr.

To hydrolyze non-annealed ssDNA regions, 1 ml of S_1 nuclease digestion buffer (0.3 M NaCl, 0.05 M acetic acid, 0.5 mM $ZnCl_2$, pH 4.6 [47]); 100U of S_1 nuclease (Invitrogen life technologies, Rockville, MD, Catalog No. 1119737) diluted 1:100 in S_1 nuclease storage buffer (20 mM Tris-HCl, 50 mM NaCl, 0.1 mM $ZnCl_2$, 50% glycerol, pH 7.5 [47]); and 50ul of salmon sperm ssDNA (200 ng/ul as excess enzyme substrate) were added to each reaction and incubated for one hour at 50 °C. To precipitate reassociated, dsDNA, 50ul of native salmon sperm

Table 2. Percent DNA relatedness determined by the S_1 nuclease method among *Xylella fastidiosa* strains reassociated at high stringency (T_m-8 °C) with ^{33}P-labeled probe DNAs.

Unlabeled (Testor) DNAs (Host of origin and strain)		% Relative Annealing of ^{33}P-labeled DNA at T_m-8 °C						
		^{33}P-labeled probe DNAs (Host of origin and strain)						
		Grape 50025[a]	Almond 50033	Plum 50039	Plum 50016	Peach 50032	Sycamore 50059	Citrus 50031
		TAXON A (Internal relatedness, 85%)[b]						
Grape (*Vitis vinifera*)	50025	**100**[c]	99	62	53	62	—	55
	50023	78	82	—[d]	55	64	55	41
	50028	86	87	62	—	61	56	44
	50030	78	84	62	—	61	63	47
	50035	89	87	59	—	62	—	40
	50036	78	82	—	57	56	61	43
	50040	88	81	59	—	55	61	45
	50043	87	89	60	—	61	49	49
	50047	81	95	64	60	59	63	34
Alfalfa (*Medicago sativa*)	50037	87	87	60	—	65	60	42
	50038	87	97	65	57	65	66	44
	50068	81	87	64	—	65	58	48
Almond (*Prunus dulcis*)	50033	78	**100**	58	64	52	52	44
	50046	84	92	63	63	53	62	43
Maple (*Acer* spp.)	50056	78	83	56	54	59	53	51
Taxons A:B Reciprocal DNA relatedness, 58%[e]				**TAXON B** (Internal relatedness, 84%)				
Peach (*Prunus persica*)	50032	48	47	86	100	**100**	93	46
Elm (*Ulmus* spp.)	50063	54	58	87	—	76	81	56
Plum (*Prunus domestica*)	50039	59	51	**100**	77	85	84	43
	50016	48	41	75	**100**	87	80	38
Grape (*Vitis aestivalis*)	50054	43	44	—	73	74	77	47
Sycamore (*Platanus* spp.)	50059	55	52	80	98	96	**100**	50
Almond (*P. dulcis*)	50045	58	65	77	—	77	78	48
Taxons B:C Reciprocal DNA relatedness, 45%						**TAXON C** (Internal relatedness, 87%		
Citrus (*Citrus* spp.)	50031	34	24	27	55	49	46	**100**
	50024	43	30	47	54	—	45	84
	50066	35	25	20	38	56	42	90
Taxons A:C Reciprocal relatedness, 41%								

[a]Strains from International Collection of Phytopathogenic Bacteria, Ft. Detrick, MD.
[b]Mean internal% DNA relatedness calculated from heterologous pairwise tests (non-bolded figures) within the taxon (boxed figures) but excluding 100% homologous values.
[c]Controls: Homologous tests (bolded figures) between probe and testor DNAs from the same strain are set to 100% DNA relatedness; heterologous tests between probe and salmon sperm (not shown) are set to 0% DNA relatedness.
[d]—, Pairwise test not performed.
[e]Mean reciprocal % DNA relatedness calculated from pairwise, heterologous tests between two taxons.

dsDNA (1.2 ug/ul as precipitation nuclei) and 0.5 ml of cold (4 °C) HCl precipitation solution (1 M HCl, 1% $Na_4P_2O_7$, 1% NaH_2PO_4) were added to each tube, mixed by vortexing, and incubated for one hour at 4 °C. Precipitated dsDNA (ca. pH 1.0) was impacted by vacuum on fiberglass filter circles (Millipore, Catalog No.APFF02500) and rinsed twice with 0.2X HCl precipitation solution to remove labeled nucleotide base digestion products. The filters were dried at 55–60 °C for 30–60 min and radioactivity was measured as counts per minute (cpm) on a LS 6500 Scintillation Counter (Beckman Instruments Inc., Columbia, MD). Separate hybridizations using labeled ssDNA as probe to salmon sperm ssDNA or homologous bacteria nonlabeled ssDNA, were included in each experiment as negative (0%) and positive (100%) controls, respectively. For the background or 0% control, data for homologous and heterologous reassociations were corrected by subtracting any cpm values from any apparent hybridization between labeled DNA and salmon sperm ssDNA (200ng/ul). The percent DNA relatedness was calculated by dividing the cpm of the heterologous reassociations by the cpm of the homologous DNA (100% control) [47]. Reactions were run at least twice and results recorded as a mean value.

DNAs from the following six strains were labeled and used as probes: grape strain ICPB 50025 (ATCC 35879^T); almond strain ICPB 50033; plum strains ICPB 50016 and 50039; peach strain ICPB 50032; sycamore strain ICPB 50059; and citrus strain ICPB 50031. DNAs from 25 strains were reassociated with the probe DNAs (Table 2).

Intergenic spacer region (ITS). Direct PCR amplification of the ITS fragment between the 16S and 23S rRNA genes was carried out using universal *Escherichia coli* primers 1493f and 23r, as described [5, 56, 83], except a 9700 Sequence Detection System (Applied Biosystems Inc., Foster City, CA) was used. The amplified products were purified using a commercial kit („Wizard DNA Clean-Up System", Catalog#A7280, Promega, Madison, WI) according to the manufacturer protocol and directly sequenced using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Norwalk, CT) and an ABI 310 Capillary Sequencing Apparatus according to the manufacture (Applied Biosystems, Foster City, CA). Nucleotide sequences were checked and edited against their electrophoregrams with Sequence Navigator 1.01 program (Applied Biosystems) and compared with Gene Inspector 1.5 *f* program (Textco, Inc., Research Triangle Park, NC). Fourteen of the 25 strains used for the DNA-DNA hybridization experiments and one additional strain from citrus (FK-83) were sequenced.

Results

DNA-DNA relatedness

At T_{spp}, mean DNA relatedness values among all DNAs from *X. fastidiosa* strains were ≥ 70% (results not presented). At T_{sub}, DNA relatedness values for heterologous reassociations ranged from a high of 99% for a labeled almond strain (ICPB 50033) and unlabelled grape strain (ICPB 50025) to a low of 20% for labeled plum (*P. domestica* L.) strain (ICPB 50039) and unlabelled citrus strain (ICPB 50066 (Table 2). Based upon a recommended relatedness value of 70% DNA relatedness for

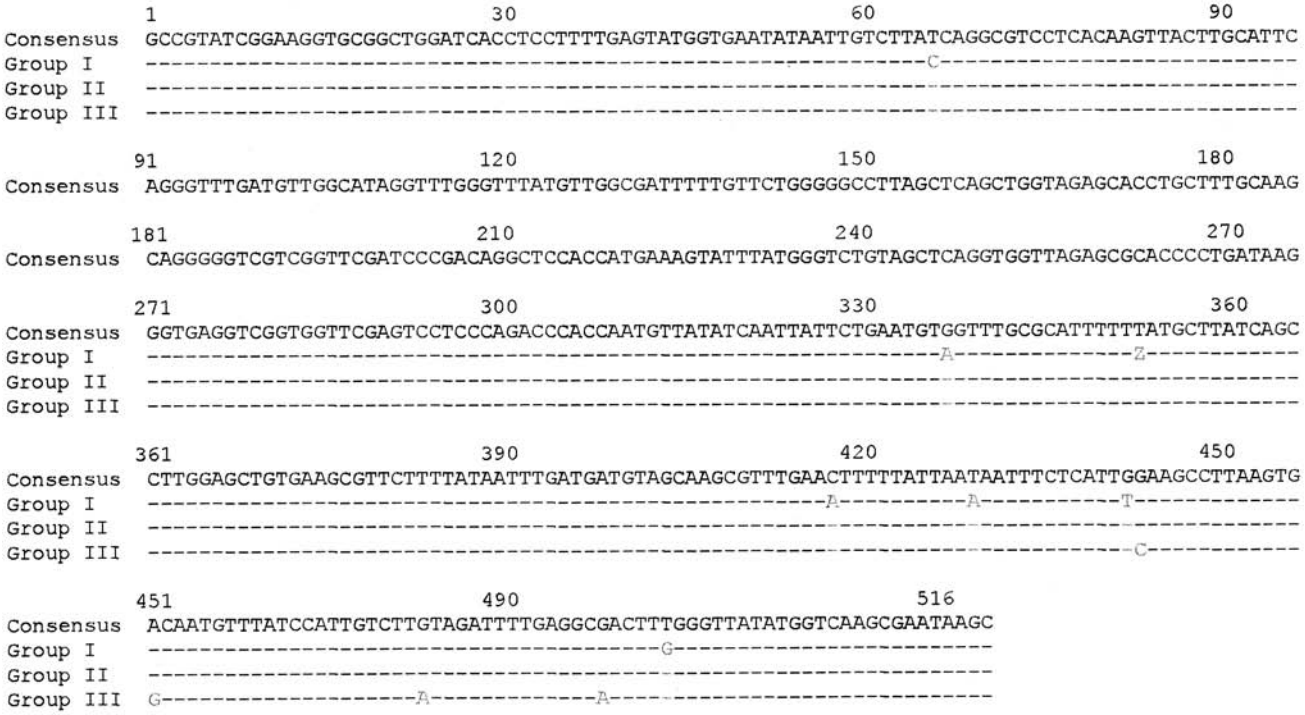

Fig. 1. Intergenic spacer region (ITS) nucleotide base sequences of *Xylella fastidiosa* strains. The general consensus for 15 strains is shown for all bases (1 to 516). Consensus sequences representing Groups I (strains 50025, 50036, 50033, 50037, 30046, 50056), II (strains 50016, 60032, 50039, 50045, 50054, 50059, 50063), and III (strains 50031, 50082) are shown only for those bases (A, C, G, T) differing or deleted (Z) from the general consensus.

Table 3. Characters useful for differentiating subspecies of *Xylella fastidiosa*.

Character	Subspecies		
	piercei	*multiplex*	*pauca*
DNA/DNA relatedness to:[1]			
piercei	85	58	41
multiplex	58	84	45
pauca	41	45	87
ITS similarity to:[1]			
piercei	100	98.7	97.9
multiplex	98.7	100	99.2
pauca	97.9	99.2	100
Growth on:[2]			
PD2 medium	++	+/–	+/–
PW medium	++	++	+
Susceptibility to:			
Penicillin	low	high	high
Carbenicillin	medium	low	low
ELISA, antisera to:[3]			
piercei	+++	+	ND
pauca	+	+++	ND
Hosts	Grape, almond, alfalfa, maple	Peach, plum, almond, elm sycamore, pigeon grape	citrus

[1] Figures are mean percent

[2] +/–, very slow growth, 10–12 days for visible colonies; +, slow growth, 8–10 days for visible colonies; ++, relatively fast growth, 5–7 days for visible colonies; PD2, Pierce's disease medium; PW, periwinkle medium; taken from Hopkins (40).

[3] Relative intensity; +, weak; +++, strong; taken from Hopkins [40]; serology tests differentiate subsp. *pauca* from subsp. *piercei* and subsp. *multiplex* [31, 54].

establishing species [79], the 25 strains of *X. fastidiosa* can be divided into three distinct taxons.

Taxon "A": Contains grape, alfalfa (*Medicago sativa* L.), almond, and maple (*Acer* spp.) strains. The 15 taxon A strains shared a mean internal DNA relatedness of 85% (range of 78–100%). Taxons A and B and taxons A and C shared a mean reciprocal DNA relatedness of 58% and 41%, respectively (Table 2).

Taxon "B": Contains peach, plum, almond, sycamore (*Platanus* spp.), elm (*Ulmus americana* L.) and pigeon grape (*Vitis aestivalis* L.) strains. These seven strains shared a mean internal DNA relatedness of 84% (range 73–100%) and a mean reciprocal DNA relatedness with taxons A and C of 58% and 45%, respectively (Table 2).

Taxon "C": Contains only citrus strains. The three citrus strains shared a mean internal DNA relatedness of 87% and a mean reciprocal DNA relatedness with taxons A and B of 41% and 45%, respectively (Table 2).

ITS sequence comparisons

The primers amplified a region of approximately 520 nucleotides. Based upon sequence differences, the 15 strains could be divided into three groups (Fig. 1). All strains within each group had identical sequences.

Group I contains grape strains (ICPB 50025 and 50036), almond strains (ICPB 50033 and 50046), alfalfa strain ICPB 50037, and maple strain ICPB 50056. Group II contained plum strains (ICPB 50016 and 50039), peach strain ICPB 50032, pigeon grape strain ICPB 50054, elm strain ICPB 50063, sycamore strain ICPB 50059, and almond strain ICPB 50045. Group III contained only citrus strains (ICPB 50031 and 50082).

Strains of group I showed similarities of 98.7% (seven nucleotides different) and 97.9% (11 nucleotides different) with strains of group II and III, respectively. Strains of Group II shared similarities of 98.7% (seven nucleotides different) and 99.2% (four nucleotides different) with strains of Group I and III, respectively. Strains of Group III shared similarities of 97.9% (11 nucleotides different) and 99.2% (4 nucleotides different) with strains of Group I and II, respectively.

Discussion

Currently, all *X. fastidiosa* strains are classified into a single species based on the original description [80]. Many studies have indicated phenotypic [12, 13, 21, 40] and genetic differences [1, 4, 15, 16, 17, 18, 20, 22, 34, 52, 61, 65, 69] suggesting phylogenetic diversity within the species. Pooler and Hartung [65] distinguished five groups within *X. fastidiosa* based on random amplified polymorphic DNA (RAPD) PCR analysis: 1) CVC, 2) plum-elm, 3) grape-ragweed (*Ambrosia arstemissiifolia* L.), 4) almond, and 5) mulberry (*Morus* spp.). Using restriction fragment length polymorphism (RFLP) analysis, Chen et al. [16] observed "striking genetic uniformity" among all 16 strains from grape. Based on differences in ITS sequencing, Hendson et al. [34] distinguished four groups: 1) plum, peach, and almond (9 of 12); 2) oak (*Quercus* spp.); 3) oleander (*Nerium oleander* L.); and 4) grape, almond (3 of 12), alfalfa (*Medicago sativa* L.), and maple. They distinguished five groups using repetitive extragenic palindromic (REP)-PCR fingerprinting and RAPD-PCR analysis by further separating the peach/plum strains from the almond strains. Hendson et al. concluded that each of the genetic groups, if supported by DNA relatedenss data, should be considered as distinct species. The DNA-based results of Hendson et al. [34] support earlier suggestions that although strains of *X. fastidiosa* constitute a single species-level taxon they contain considerable genetic diversity. Using a spectrophotometric method [26] to assay DNA relatedness, Kamper et al. [49] showed that plum strains shared 85–90% DNA relatedness with peach and periwinkle (*Vinca major* L.) strains but only 75% relatedness with grape strains. Based on these differences, the authors suggested that the plum/peach/periwinkle strains constituted a subspecies different from the grape strains. In this study,

we used the more conservative S_1 nuclease method [45, 47] recommended for more robust phylogenetic analyses, [75, 76, 77] to determine DNA relatedness and re-examine the differences observed by Kamper et al. [49].

Using a stringent temperature of 62 °C (T_m –15 °C), our results (not presented) of DNA-DNA relatedness agreed with those of Kamper et al. [49]. All strains were related at a value of 70% or greater. Although the relatedness values for the spectrophotometric method are similar, they are not directly comparable [47]. With the spectrophotometric method, homologeous as well as non-homologous annealing occurs often resulting in abnormally higher DNA relatedness values. Therefore, the spectrophotometric system relies upon an algorithum to determine the portion of the heterologous DNA among a mixture of double-stranded homologous DNAs and single stranded DNAs [45, 47]. With our S_1 nuclease method, labeled probe DNA is only a small fraction (1:500 to 1:700) of the concentration of the testor DNA, practically eliminating homologous re-annealed DNA.

Under standard reassociation conditions (T_m–15 °C to T_m–25 °C), the percent DNA relatedness of DNA duplexes resulting from homologous re-annealing are not significantly different [9, 48]; however, over this same temperature range, duplexes formed between heterologous DNAs are significantly less stable [9, 48]. The thermal stability of nucleic acid duplexes is sensitive to the presence of mismatched nucleotide pairs within the polymer strands [2, 19, 50], variations in genome size [19], and quality of extracted DNA [19]. Because heterologous heteroduplex molecules from closely related organisms are less stable than homologous duplex molecules over the same range of conditions [8, 9, 10, 11, 48], we reasoned that use of an even more stringent reassociation temperature (82.4 °C = T_m–8 °C; with 22.7% formamide = 68.8 °C) might magnify the nucleotide base sequence differences among closely related *X. fastidiosa* strains and result in more reliable reciprocal results. Indeed, using this highly stringent temperature, our DNA-hybridization results revealed clear and repeatable differences among strains of *X. fastidiosa* at the species or sub-species level and allowed differentiation of three distinct taxons. At high stringency (T_m –8 °C), reassociations among homologous (more complementary) sequences from phylogenetically more closely related *X. fastidiosa* strains are more stable while reassociations among heterologous DNAs from less phylogenetically related *X. fastidiosa* strains are less stable and, therefore, have lower % DNA relatedness values.

Our ITS sequencing results agreed closely with our DNA-DNA relatedness assays and the differences between the three taxons were consistent between the two methods. The three taxons differed by up to 11 nucleotides out of 520 nucleotide base pairs sequenced (Fig. 1). Our ITS results are in close agreement with the ITS results of Hendson et al. [34]. We used three strains (ICPB 50040, 50043, and 50056) that Hendson et al. [34] used and they all segregated in both DNA relatedness and ITS assays in the same manner reported previously [34]. In contrast, in another study using ITS sequencing [61], two groups were differentiated; one containing citrus, coffee, peach, plum, and oak strains and a second containing grape, maple, and oleander strains [61]. Our ITS sequence assays agree with the latter group but our results separate peach and plum strains from the citrus strains. RAPD-PCR, RFLP, and PFGE results divided the strains into several additional groups [34]. These results show that RAPD, pulse field gel electrophoresis (PFGE), and RFLP assays are more useful for identifying strains than are DNA relatedness assays and ITS sequencing. To further support this point, differences among citrus strains (CVC) and other strains of *X. fastidiosa* have been revealed by RAPD analyses [65], analyses of tandem repeats [18], and PCR [20]. Citrus and coffee (*Coffee arabica* L.) strains were clearly separated from grape strains based on arbitrarily PCR [20]. Thus it is clear from the many molecular studies that *X. fastidiosa* consists of several genetically distinct groups of organisms that should be recognized as separate taxons within the species *X. fastidiosa*.

To create a new species, normally phenotypic tests should be found that correlate with DNA relatedness groupings [11]. Because *X. fastidiosa* is a fastidious organism, its metabolism has not been studied and so no definitive biochemical tests are available to differentiate the three taxons. However, in general, the available phenotypic and serological analyses agree favorably with the molecular data presented here and reviewed above. Growth characteristics on media designed for *X. fastidiosa* are well known [12, 14, 21, 40, 78]. Group A (grape, alfalfa, maple, and almond) strains grow well on most media designed for *X. fastidiosa* including PD2, PW, buffered charcoal yeast extract (BCYE), and Chang and Schaad 20 [CS20] [40] whereas strains from taxon B (peach, plum, sycamore) and Taxon C (citrus) grow much slower on these media. The taxon B [23] and C [21, 57] strains grow best on PW. Taxon B and C strains are susceptible to penicillin and resistant to carbenicillin whereas the opposite is true for Taxon A strains [40]. Strains of taxons A and B and C can be differentiated serologically [31, 40, 54]. The type strain from grape (ATCC 35879) is clearly differentiated from strains from peach, plum, and periwinkle by protein profiles [13]. One might not expect that pathogenicity of an organism that causes disease in such a large number of different plant families would be reliable as a criterion for classification. However, pathogenicity is in many cases a useful, reliable phenotypic character for identifying *X. fastidiosa* sub-taxons. All grape and alfalfa strains belong to taxon A, all peach and plum strains belong to taxon B, and all citrus strains belong to taxon C.

Similar to the wide host range bacterium *Pseudomonas syringae* pv. *syringae* (van Hall 1902 Dye et al., 1980) [27], *X. fastidiosa* causes diseases with varied symptomologies in many different plants [39, 67]. Although DNA relatedness and ITS sequence data show strains of *X. fastidiosa* from grape and alfalfa are genetically in the same taxon, symptoms of alfalfa dwarf "differ sharply from Pierce's disease" [http://www.cnr.berkeley.edu/xylella/calif.html]. However, cross inoculation

with strains causing Pierce's disease, almond leaf scorch, and alfalfa dwarf showed that the varied symptoms were all caused by the same strain of *X. fastidiosa* [24, 25, 78]. These results support observations that "the incidence of PD in vineyards is typically highest adjacent to alfalfa fields with alfalfa dwarf disease" [42]. The same is true for the genetically similar peach and plum strains; symptoms on plum are observed primarily as a leaf scorch and never as a dwarfing symptom as observed in peach [75]. Successful cross inoculation of peach and plum suggested the similarity of these organisms [21]. Among the strains we tested, only *X. fastidiosa* strains isolated from almond were present in two different taxons. Almond strain ICPB 50045 grouped together with taxon B (plum, peach, sycamore, pigeon grape, and elm strains) and not with almond strains ICPB 50033 and 50046 in taxon A. These results are in agreement with the results of Hendson et al. [34]. The presence of almond strains in both DNA hybridization taxons A and B might suggest cross-infectivity with peach, however, phony peach is not commonly observed in California yet peaches are observed growing side-by-side with grapes infected with Pierce's disease. Also, inoculation experiments of peach seedlings with strains of *X. fastidiosa* isolated from grape and almond seedlings with strains from peach were unsuccessful [81]. In contrast, Li et al. [57] showed that the genetically different citrus strains caused leaf scorch symptoms in grape when grapevines were inoculated. For inoculation, they used a large gauge (20 g) needle and an undiluted seven day-old liquid culture containing 10^8 to 10^9 cfu/ml, as recommended for routine pathogenicity tests with *X. fastidiosa* [40]. However, for cross inoculation studies of such broad host pathogens as *P. syringae* pv. *syringae* [37] and *X. fastidiosa*, special care must be taken in the determination of pathogenicity to avoid reactions on non-hosts that may be misinterpreted as a true pathogenic response. For such organisms it is recommended to use a natural means of inoculation and a relatively low inoculum level containing from 10^3 to 10^5 cfu/ml [37]. Although symptoms would take additional weeks or months, the results would be more reliable and meaningful. This would be especially important for the citrus strain (CVC) because it is highly regulated and included on the APHIS Select List of Pathogens [http://www.aphis.usda.gov/ppq/permits/bioterrorism/] whereas the Pierce's disease bacterium is not.

The two elm and sycamore strains grouped together in taxon B, however, cross inoculation studies showed neither strain to be pathogenic on the other host [72]. This might suggest the presence of pathovars, however, DNA relatedness assays should be conducted with additional strains.

Following annealing (T_m –15 °C to T_m –25 °C), ≥70% DNA similarity is accepted to delineate strains of the same species [45, 79]. In this study, we could not differentiate among many *X. fastidiosa* strains at T_m –15 °C but we could place strains into three clearly separate taxons, A, B, and C using a T_m of –8 °C. This shows that these taxons can be separated at the subspecies level; and should be classified as separate subspecies. Strength is lent to this argument because DNA hybridization taxons A, B, and C correlated very well with ITS taxons I, II, and III, which shared identical sequences inside taxons and had identical transitions of nucleotides between them. Furthermore, phenotypic, serological and pathogenic characters correlated and are useful for identification.

Therefore, we propose taxons A, B, and C be classified as subspecies and named *X. fastidiosa* subsp. *piercei*, *multiplex*, and *pauca*, respectively. We previously [71] proposed "*agglomeri*" and "*idiotroposa*" for group B and C strains, respectively. However, we believe the names "*multiplex*" (meaning many) and "*pauca*" (meaning few) better describe these bacteria.

Summary of characters. Table 5 summarizes some of the most important characters for distinguishing *X. fastidiosa* subspecies *piercei*, *multiplex*, and *pauca*.

Species description: The description of the species *Xylella fastidiosa* remains unchanged with the type strain ATCC 35879.

Xylella fastidiosa* subsp. *piercei (pier' ce.i. L. gen. masc. n. piercei of Pierce; named in honor of N.B. Pierce, who first described a disease (Pierce's disease of grape, *V. vinifera*) caused by the bacterium). Strains of *X. fastidiosa* subsp. *piercei* grow faster on the following media: PD2, PW, BCYE, and CS20, are more resistant to penicillin and less resistant to carbenicillin, than *X. fastidiosa* subsp. *multiplex and pauca*. Serology differentiates *X. fastidiosa* subsp. *piercei* from *X. fastidiosa* subsp. *multiplex* and *X. fastidiosa* subsp. *pauca*. Protein profiles of *X. fastidiosa* subsp. *piercei* are distinct from *X. fastidiosa* subsp. *multiplex*. DNA similarity assays and ITS sequence assays separate all three subspecies, *piercei*, *multiplex*, and *pauca*. This bacterium causes disease in grape (*V. vinifera* L.), alfalfa (*Medicago sativa* L.), maple (*Acer* spp.), and almond (*P. dulcis* Mill., L.). Symptoms vary from host to host but include leaf scorch, veinal chlorosis, wilt, and dwarfing. Cross inoculation with subsp. *piercei* strains from the above hosts generally results in pathogenicity, suggesting a lack of any pathovars. Type strain: ATCC 35879 (ICPB 50025). A culture has been deposited in the International Collection of Micro-organisms from Plants (ICMP), Auckland, New Zealand as ICMP 15197.

Xylella fastidiosa* subsp. *multiplex (mul' ti. plex. L. adj. multiplex, numerous/ manifold; named to recognize the large number of host plants in which the bacterium causes disease). Known hosts include peach (*P. persea* L. Batsch), plum (*P. domestica* L.), almond (*P. dulcis* Webb L.), elm (*Ulmus* spp.), pigeon grape (*V. aestivalis* Michx.), sycamore (*Platanus* spp.), and other shade trees. Strains of *X. fastidiosa* subsp. *multiplex* grow much faster on PW medium than on PD2, BCYE, or CS20, are more susceptible to penicillin, and more resistant to carbenicillin, than *X. fastidiosa* subsp. *piercei*. Serology differentiates *X. fastidiosa* subsp. *multiplex* from *X. fastidiosa* subsp. *piercei* and *X. fastidiosa* subsp. *pauca*. Protein profiles of

X. fastidiosa subsp. *multiplex* are distinct from *X. fastidiosa* subsp. *piercei*. DNA similarity assays and ITS sequence assays separate all three subspecies, *piercei*, *multiplex*, and *pauca*. Symptoms vary from host to host but include leaf scorch, veinal chlorosis, wilt, and dwarfing. Cross inoculation with strains from different hosts do not always result in pathogenicity, suggesting that pathovars with restricted host ranges may exist. Type strain: ATCC 35871 (ICPB 50039). A culture has been deposited in ICMP as ICMP 15199.

Xylella fastidiosa* subsp. *pauca (pau' ca L. fem. adj. pauca few; named to recognize the narrow host range of this bacterium). Only strains of *X. fastidiosa* subsp. *pauca* cause citrus veinal chlorosis. *X. fastidiosa* subsp. *pauca* and *multiplex* grow more slowly than *X. fastidiosa* subsp. *piercei* on the following media: PD2, PW, BCYE, and CS20, are more susceptible to penicillin, and more resistant to carbenicillin than *X. fastidiosa* subsp. *piercei*. *X. fastidiosa* subsp. *pauca* can be differentiated from the other two subspecies by differences in susceptibility to antibiotics. Serology can differentiate *X. fastidiosa* subsp. *pauca* from *X. fastidiosa* subsp. *multiplex* and subsp. *piercei*. DNA relatedness assays and ITS sequence assays separate all three subspecies, *piercei*, *pauca*, and *multiplex*. Pathogenic to citrus (*Citrus* spp.); symptoms include veinal chlorosis and smaller fruit. Type strain: ICPB 50031 (09-02N). A culture has been deposited in ICPM as ICPM 15198.

All strains are available in the International Collection of Phytopathogenic Bacteria (ICPB) maintained by ARS, Ft. Detrick, MD (N.W. Schaad, Curator).

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